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ARGININE-RICH ANTI-VASCULAR ENDOTHELIAL GROWTH FACTOR

PEPTIDES THAT INHIBIT GROWTH AND METHASTASIS OF HUMAN

TUMOR CELLS BY BLOCKING ANGIOGENESIS

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FIELD OF THE INVENTION

The present invention relates to a novel peptide that shows inhibitory activity of the vascular endothelial growth factor (hereinafter, referred to as "VEGF"), which is an angiogenic factor, and the use thereof for the prophylaxis and treatment of cancers and angiogenesis-related diseases.

BACKGROUND OF THE INVENTION

With a definition for the generation of new blood vessels in adult tissues, but not for the vasculogenesis during embryogenesis or development, angiogenesis is a biological process in which angiogenic factors, substrate molecules and accessory cells are elaborately synchronized in time and space. The generation of blood vessels is achieved in complex, collective, multi-step bioreactions, playing a very important role in normal physiological functions, such as wound healing, embryogenesis, etc. In the body, angiogenesis is conducted at a necessary time in a necessary place for a required period under an elaborate system controlled by the balance between angiogenic factors and antiangiogenic factors (Loitta, L. A. et al., Cell, 64,

327 (1991)).

A failure in controlling the elaborate mechanism of angiogenesis results in various diseases, including cancers, diabetic retinopathy, rheumatoid arthritis, etc (Kohn, E. C. et al., Proc. Natl. Acad. Sci. USA, 92, 1307 (1995); Folkman, J. et al., Science, 235, 442 (1997); Risau, W., Nature, 386, 671 (1997)). Also, angiogenesis is revealed to be indispensable for the growth and metastasis of cancer cells because it enables nutrients to be provided to cancer cells and makes passageways through which cancer cells are transferred to other sites (Hanahan, D. et al., Cell, 86, 353 (1996); Skobe, M. et al., Nature Med., 3, 1222 (1997)). In detail, cancerous cells grow to the size of 2 mm or larger, new blood vessels are formed around the tumor through which the supply of oxygen and nutrients and the removal of waste products are allowed (Fidler, I. J. et al., Cell, 79, 185 (1994)). In addition, the metastasis of cancerous cells can be accelerated through the vast capillary networks newly formed by various angiogenic factors secreted from cancerous cells or normal tissue cells (Blood, C. H. et al., Biochem. Biophys. Acta., 1032, 89 (1990)).

A limitation of conventional anticancer agents and chemical therapies is that various types of cancerous cells are present even in a single tumor and they have varying mutation and growth rates that are higher than those of normal cells and consequently they become resistant to the

conventional anticancer agents. In contrast, anti-angiogenic therapies for cancer inhibit the growth of host normal cells (vascular endothelial cells), but not cancerous cells themselves, so that they are expected to overcome the problems of conventional therapies for cancer, which are due to the versatility and resistance of cancerous cells. Advantages of the antiangiogenic therapies to preexisting therapies for cancer is supported by various animal test results published by many researchers (Burrows, F. J. et al., Pharmac. Ther., 64, 155 (1994)).

In the body coexist angiogenic factors and antiangiogenic factors, through the balance of which angiogenesis is elaborately performed. Until now, there have been known dozens of cancer-relevant angiogenic factors, most of which do not act as growth factors for endothelial cells (Bussolino, F. et al., Trends. Biochem. Sci. 22, 251 (1997)). On the other hand, VEGF is known to act as an endothelial cell-specific growth factor *in vitro* (Gospodarowics, D, et al., Proc. Natl, Acad. Sci., USA, 86, 7311 (1989)), increases vascular permeability (Leung, D. W. et al., Science, 246, 1306 (1989), and induces the angiogenesis related to the progress of cancer *in vivo* (Plouet, J. et al., EMBO J., 8, 3801 (1989)).

It is revealed that VEGF is one of the most potent, angiogenic factors, whose expression is induced by a variety of stimuli, including hypoxia, and is indispensably required for the growth and metastasis of human cancerous cells in

vivo (Connolly, D. T. et al., J. Biol. Chem. 264, 20017 (1989); Kim, K. J. et al., Nature 362, 841 (1993)). VEGF binds to heparin and shares homology in amino acid sequence with PLGF (placental growth factor) and PDGF (platelet-derived growth factor) (Conn, G. et al., Proc. Natl. Acad. Sci., USA, 87, 2628 (1990); Keck, P. I. et al., Science, 246, 1309 (1989); Maglione, D. et al., Proc. Natl. Acad. Sci., USA, 88, 9267 (1991)). Also, it is known that VEGF is expressed as four isoforms consisting of 121, 165, 189, and 206 amino acids, respectively, by alternative splicing (Tischer, E. et al., J. Biol. Chem., 266, 11947 (1991)), of which the VEGF₁₂₁ is not associated with heparin.

The signal transduction pathway of VEGF by which it exerts its functions as a growth factor starts with the binding of VEGF to cellular receptors (KDR/Flk-1 and Flt-1) which are specifically expressed on vascular endothelial cells (Millauer, B. et al., Cell, 72, 835 (1993), De Vries, C., et al., Science 255, 989 (1992)). The significance of VEGF in vasculogenesis during embryogenesis and in angiogenesis has been demonstrated by gene deletion studies of VEGF and VEGFR, (Fong, G. h. et al., Nature, 376, 66 (1995); Shalaby, F. et al., Nature, 376, 62 (1995); Carmeliet, P. et al., Nature, 380, 435 (1996); Ferrara, N. et al., Nature, 380, 439 (1996)), the malignant transformation of cancerous cells upon over-expression of VEGF in cancer cells (Zang, H. T. et al., J. Natl. Cancer Inst., 87, 213 (1995)), and the inhibition of the growth of

cancer cells by neutralizing anti-VEGF monoclonal antibodies and by the expression of soluble Flt-1 and dominant-negative KDR/FlK-1 (Kim, K. J. et al., Nature, 362, 841 (1993); Goldman, C. K. et al., Proc. Natl. Acad. Sci., USA, 95, 8975 (1998)). In addition, there are reported other research results which prove that VEGF plays a key role in angiogenesis.

Therefore, materials which act to inhibit the association between VEGF and its receptors can suppress the angiogenesis driven by VEGF as well as the growth and metastasis of cancer cells, which secrete VEGF (Martiny-Baron, G. et al., Curr. Opin. Biotechnol., 6, 675 (1995)).

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a VEGF-antagonistic peptide which is able to inhibit angiogenesis and the growth and metastasis of cancer cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1a is a curve showing that ^{125}I -labeled VEGF binds to its receptors on the surface of HUVEC (human umbilical vein endothelial cells) in a time-dependent manner.

Fig. 1b is a curve showing that ^{125}I -labeled VEGF binds to its receptors on the surface of HUVEC in a dose-dependent manner.

Fig. 1c is a Scatchard plot obtained from the results of Fig. 1b, which shows that two kinds of VEGF receptors exist on the surface of vascular endothelial cells.

Fig. 2 provides histograms showing a primary searching results, in which the inhibitory activity of combinatorial peptide libraries against the binding of VEGF to its receptors is represented according to amino acid residues on each position of the hexa-peptide when combinatorial peptide libraries are used at a concentration of 0.33 nM/peptide.

Fig. 3a is a histogram in which the inhibitory activity of the first secondary pool of peptides, synthesized on the basis of the primary searching results, against the binding of VEGF to its receptors is represented according to amino acid residues when the pool is used at a concentration of 100 nM/peptide.

Fig. 3b is a histogram in which the inhibitory activity of the second secondary pool of peptides, synthesized on the basis of the results of Fig. 3a, against the binding of VEGF to its receptors is represented according to amino acid residues when the pool of peptides is used at a concentration of 250 nM/peptide.

Fig. 4 shows curves in which the inhibition activity of six fractions separated from a mixture of the most effective peptides selected from the second secondary pool through a C_{18} reverse-phase column according to the retention time. The Inhibitory activity of each fraction against the binding of VEGF to its receptors, is plotted

versus concentration of the fractions.

Fig. 5 shows curves in which the inhibitory activity of 12 peptides against the binding of VEGF to its receptors is plotted versus peptide concentration.

5 Fig. 6 is a histogram showing quantitative results for the binding of I^{125} -labeled VEGF and I^{125} -labeled bFGF (basic fibroblast growth factor) to their receptors in the presence of selected three peptides or other control peptides.

10 Fig. 7 shows curves in which radioactivity measured from I^{125} -labeled VEGF associated with fixed peptides (Sequences 1, 2, and 3) is plotted versus molar ratios of competitors (excess free peptides) to their counterparts.

15 Fig. 8 is a histogram showing radiation quantities measured from I^{125} -labeled VEGF associated with a fixed sequence (Sequence 1) in the absence of and in the presence of competitors (excess non-labeled VEGF, free Sequences 1, 2, and 3).

20 Fig. 9 provides histograms in which the radioactivity measured from I^{125} -labeled VEGF associated with fixed Sequence 1 (RRKRRR) is plotted in the absence of competitors and in the presence of competitors for various VEGF isoforms.

Fig. 10a provides curves showing that the peptides of Sequences 1, 2, and 3 inhibit the VEGF-induced growth of vascular endothelial cells in a dose-dependent pattern.

25 Fig. 10b is a histogram in which the radioactivity of the thymidine incorporated into the genome of HUVEC is measured in the absence of peptides and in the presence of

100 μ M of each of the three peptides, demonstrating that the inhibition of cell growth is not attributed to the cytotoxicity of the peptides.

Fig. 11 gives photographs of rabbit corneal domes showing the obvious angiogenesis in the test groups treated with VEGF only (A), the anti-angiogenic effect in the test group treated simultaneously with both Sequence 1 RRKRRR and VEGF (B), and the obvious angiogenesis in the test group treated with the peptide EEFDDA (C).

Fig. 12 gives photographs showing the inhibitory effect of Sequences 1, 2, and 3 on the angiogenesis induced by VEGF secreted from human cancer cells.

Fig. 13 shows curves in which viability of human fibro sarcoma cell line is plotted versus concentrations of peptides, demonstrating that the peptides of the invention have no direct influence on human fibro sarcoma cells.

Fig. 14 shows curves in which changes in tumor size are recorded over the time period of injection, demonstrating that the peptide of Sequence 1 effectively inhibits the growth of human colon carcinoma cells in mice.

Fig. 15a is a histogram in which the numbers of metastatic nodules from spleen to liver are measured after treatment with saline solution and with various peptides.

Fig. 15b is a histogram in which the weights of mouse livers to which the human colon carcinoma cells are transferred from the spleen are measured after treatment with various peptides.

DETAILED DESCRIPTION OF THE INVENTION

To separate new anti-VEGF peptides in the present invention combinatorial library of small peptides were used, from which the development of small molecule drug candidates has proven remarkably successful (Gho, Y. S. et al., Cancer Res., 57, 3733 (1997); Park, J. Y. et al., Endocrinology, 138, 617 (1997)).

To begin with, a peptide combinatorial library is constructed. In this regard, there are synthesized hexapeptides in which 19 kinds of amino acids are specified at each amino acid position. If a specific amino acid residue at a determined position and other amino acid residues at the other positions in the hexa-peptides are represented by O and Xs, respectively, the peptides can be expressed as $OX_2X_3X_4X_5X_6$, $X_1OX_3X_4X_5X_6$, ... $X_1X_2X_3X_4X_5O$, which amounts to 114 (6x19) combinations.

From the combinatorial peptide library are separated the peptide sequences which inhibit at the highest efficiency the binding of VEGF to its receptors present on vascular endothelial cells. In the present invention, a radioactive iodine-labeled VEGF, along with the combinatorial peptide library, is added to a culture of vascular endothelial cells and allowed to react with one another. After the removal of unbound VEGF, quantification of the bound VEGF to the receptors present on the

endothelial cell surface is performed by measuring the radioactivity of the associated VEGF. The peptide sequences are selected which show inhibitory activity against the binding of VEGF to its receptors, at the lowest concentrations.

The soluble hexa-peptide library were tested through the selection procedure described above(Fig 2). The amino acids at each position of the hexa-peptides that show significant contribution to the inhibition are listed in Table 1, below.

<TABLE 1>

1(N)	2	3	4	5	6
K	K	K	R	K	R
R	R	R	K	R	K
G	H	H	H	W	H
A	T	T			F
H	W	W			L

Based on the results from the above primary selection, as seen in the following Table 2, peptide sequences are synthesized in such a way that the two amino acids from carboxy termini are specified by selected amino acids while the other positions are occupied by the random mixture of the amino acid residues selected through the primary selection procedure in equal ratios.

<TABLE 2>

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1-N	Mixture of K, R, G, A, H														

2	Mixture of K, R, H, T, W														
3	Mixture of K, R, W, G, S														
4	Mixture of R, K, H														
5	K	R	R	K	K	K	H	H	H	F	F	F	L	L	L
6-C	R	R	W	K	R	W	K	R	R	K	R	W	K	R	W

Again, the first sub-library is tested for the inhibitory activity against the binding of VEGF to its receptors (see Fig. 3a). According to this quantitative data, the peptide sequences which were found to inhibit the binding with the highest efficiency were those with arginine or histidine at their sixth position and arginine or lysine at their fifth position.

On the basis of the above two selection procedures, the second sub-library is constructed in such a way that the first, the fourth and the sixth positions are specified by predetermined amino acids while the other three positions are occupied by the amino acid mixtures secured through the above two selection procedures, as shown in Table 3, below.

<TABLE 3>

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1-N	K	R	G	A	H	K	R	G	A	H	K	R	A	G	H
2	Mixture of K, R, H, T, W														
3	Mixture of K, R, W, G, S														
4	R	R	R	R	R	K	K	K	K	K	H	H	H	H	H
5	Combinatorial library K, R														
6-C	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

Analysis for the inhibitory activity against the activity of VEGF reveals that the peptides in which the

first, the fourth and the sixth positions are all specified by arginine inhibit the binding of VEGF to its receptors with the most efficiency (see Fig. 3b).

Thereafter, additional analysis of the peptide mixtures, obtained after separation by use of a C₁₈ reverse-phase column, for inhibitory activity against the binding of VEGF to its receptors led to the conclusion that the most effective inhibitory activity is induced when the first, the fourth and the sixth positions all are occupied by arginine, the second position by arginine, lysine or histidine, and the third and the fifth positions by arginine or lysine (see Fig. 4).

These preferable combinations for the amino acid sequence are shown in Table 4, below.

<TABLE 4>

	1	2	3	4	5	6	7	8	9	10	11	12
1(R)-N	R	R	R	R	R	R	R	R	R	R	R	R
2(K, R, H)	K	R	H	K	R	H	K	R	H	K	R	H
3(K, R)	K	K	K	R	R	R	K	K	K	R	R	R
4(R)	R	R	R	R	R	R	R	R	R	R	R	R
5(K, R)	K	K	K	K	K	K	R	R	R	R	R	R
6(R)-C	R	R	R	R	R	R	R	R	R	R	R	R
IC ₅₀ (μM)	3.4	6.0	>20	8.5	4.5	>20	6.5	2.0	>20	7.8	3.8	>20

An examination which was made for the inhibitory activity of the 12 peptides against the binding of VEGF to its receptors verified that the peptide of sequence 1 (NH₂-Arg-Arg-Lys-Arg-Arg-Arg-CONH₂), the peptide of sequence 2 (NH₂-Arg-Lys-Lys-Arg-Lys-Arg-CONH₂), and the peptide of sequence 3 (NH₂-Arg-Arg-Arg-Arg-Arg-Arg-CONH₂) are the most

potent inhibitors against VEGF activity.

There may be various mechanisms through which the peptides can inhibit VEGF from binding to its receptors. In order to elucidate the accurate inhibitory mechanism, an examination was made of the possibility that the identified peptides could directly associate with VEGF to inhibit the binding of VEGF to its receptors present on cell surfaces of vascular endothelial cells. In this connection, labeled VEGF and non-labeled VEGF were allowed to competitively associate with immobilized peptides. From the quantification of radioactivity from the immobilized peptides, the association extent between labeled VEGF and the peptides was found to decrease with increase of the concentrations of non-labeled VEGF and free peptides added, demonstrating that the identified peptides of the present invention directly associate with VEGF. The finding that the association between a peptide and labeled VEGF is completely inhibited by all of the three peptides makes it possible to postulate that the peptides of sequence 1, sequence 2, and sequence 3 have identical or overlapped binding domains on VEGF (Figs. 7 and 8). Also, a quantitative measurement was made of the inhibitory activity of the peptide of sequence 3 against the association between 5 types of VEGF isoforms and their receptors, leading to the conclusion that both the amino and the carboxyl terminals of VEGF₁₂₁ are important to the binding domain (see Fig. 9).

In order to determine whether the peptides of the

present invention inhibit the VEGF-stimulated growth of vascular endothelial cells, the amount of DNA synthesized in vascular endothelial cells in the presence of the screened peptides and VEGF was measured. As a result, it was
5 obtained that the peptides of the present invention inhibit the DNA synthesis induced by VEGF in vascular endothelial cells in a dose-dependent pattern. In consequence, the peptides of the present invention have inhibitory activity against the VEGF-stimulated growth of vascular endothelial
10 cells (see Figs. 10a and 10b).

In an experiment, egg CAM (chorioallantoic membrane) was treated with VEGF in the presence of the peptide of the present invention to give data which show that VEGF-induced angiogenesis is inhibited by the peptides, thus verifying
15 the anti-angiogenic activity of the peptides of the present invention. Additionally, in an animal test for angiogenesis using rabbit cornea, the angiogenesis which was definitely observed from the control group treated with only VEGF was completely inhibited in the group treated with VEGF and the
20 peptides simultaneously (see Fig. 11). Further, an experiment was conducted to determine whether the peptides of the present invention are able to inhibit the angiogenesis induced by VEGF secreted from cancer cells. To this end, VEGF-secreting sarcoma was applied, along with the
25 peptides of the present invention, to egg CAM. The inhibitory effect of the peptides of the present invention on the cancer cell-induced angiogenesis was observed (see

Fig. 12).

An experiment was conducted to see whether the peptides of the invention, which were proved to have antagonistic activity to VEGF, have direct inhibitory effects on the growth of cancer cells. In the experiment, human fibro sarcoma cells were cultured and treated with the peptides of the present invention, followed by measuring cell viability. From the measurements, it is apparent that the peptides of the present invention have no direct influence on the growth of the fibro sarcoma cells, as seen in Fig. 13.

The peptides of the present invention were also investigated as to the ability to inhibit the growth and metastasis of cancer cells. Along with the peptides of the present invention, human colon cancer cells were introduced to mice by subcutaneous injection. After a certain period of time, the tumors formed were measured for size. It was observed that a significant reduction was brought about in the size of the tumor formed in the mice treated with the peptides of the present invention, compared to the control group treated with phosphate buffered saline only (see Fig. 14). In addition, when cancer cells implanted into the spleen of mice were treated with the peptides of the present invention, better results were obtained in the number of metastatic tumor nodules in liver and the weight of the liver than when the implanted cells were treated only with phosphate buffered saline (see Figs. 15a and 15b). From the

above experimental results, the peptides of the present invention can be assumed to exert their inhibitory activity against the growth and metastasis of malignant tumors by shielding the signal transduction pathway of VEGF.

5 In conclusion, through the above various experiments, it was revealed that the peptides of the present invention do not directly affect the growth of cancer cells, but specifically suppress the angiogenesis induced by cancer cells as a result of the inhibitory activity of the peptides
10 against the binding of VEGF secreted from cancer cells to receptors present on vascular endothelial cell surface.

 Thanks to superior ability to inhibit the binding of VEGF to its receptors, the peptides of the present invention can be used as therapeutics for angiogenesis-related
15 diseases, including cancer, diabetic retinopathy, rheumatoid arthritis, etc.

EXAMPLES

 Practical and presently preferred embodiments of the
20 present invention are illustrative as shown in the following Examples.

 However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of
25 the present invention.

EXAMPLE 1: Characterization of ^{125}I -labeled VEGF

Utilizing ^{125}I -labeled VEGF₁₆₅ in various experiments, an examination was made as to whether this labeled protein have the same activity as that of natural VEGF protein.

5 First, HUVEC (human umbilical vein endothelial cells) (Clonetics) were placed on gelatin-coated 100 mm dishes (Falcon) containing a vascular endothelial cell culture medium (Medium 199 + 20 % BCS, 5 $\mu\text{g}/\text{ml}$ of heparin, 6 $\mu\text{g}/\text{ml}$ of endothelial cell growth supplement, 5 ng/ml of basic
10 fibroblast growth factor) and cultured at 37°C in a CO₂ incubator for animal cell culture. After a certain period of time, the cells were treated with trypsin/EDTA. After centrifugation, the cells harvested were suspended in a fresh, vascular endothelial cells culture medium and
15 aliquoted at a density of 5×10^4 cell/well to a 24 well plate (Costar Co.). Following one-day culturing, the cells were added in a binding medium (Medium 199/25 mM HEPES, pH 7.4/0.1% bovine serum albumin) containing 0.2 ng of ^{125}I -labeled VEGF₁₆₅ and reacted at 4°C for 3 hours. To remove the
20 labeled VEGF which still remained in the medium, the cells were washed twice with the same medium as used above, and once with PBS containing 0.1% albumin. Thereafter, to quantitatively determine the amount of the labeled VEGF which bound to receptors present on the surface of the
25 vascular endothelial cells, the cells were treated with 0.5 ml of a lysis solution (20 mM Tris-HCl, pH 7.4/1% TritonX-100) for 20 min, followed by measuring the resulting lysate

for radioactivity with the aid of a γ -counter. As for the non-specific binding, it was determined from a cell group cultured in the presence of a mixture of the labeled VEGF and non-labeled VEGF in a molar ratio of 1:100. This cell group was cultured as a negative control.

The measurement results are shown in Figs. 1a and 1b in which radioactivity is plotted versus time and concentration, respectively. As seen in these figures, the binding of labeled VEGF (1 ng/ml) to VEGF receptors present on the surface of HUVEC (5×10^4 cells/well) behaves in a time-dependent pattern and a VEGF dose-dependent pattern, demonstrating that the interaction between VEGF and its receptors are specific.

Scatchard analysis of the results obtained in Fig. 1b revealed that two kinds of receptors exist on the surface of vascular endothelial cells, as seen in Fig. 1c. One of two kinds of the receptors has a dissociation constant (KD) of 3 pM and is populated at a density of about 2,000 per cells while the other has a dissociation constant (KD) of 50 pM and is populated at a density of about 6,000 per cell. These results agree with those reported from various studies (Maciag, T. et al., Proc. Natl. Acad. Sci., USA, 76, 5674 (1979); Myoken, Y. et al., Proc. Natl. Acad. Sci., USA, 88, 5819 (1991); Gengrinovitch, S. et al., J. Biol. Chem., 270, 15059 (1996); Bikfalvi, A. et al., J. Cell Physiol. 149, 50 (1991)).

EXAMPLE 2: Search from Combinatorial Peptide Library for VEGF-Antagonistic Peptide Sequence

Step 1: Construction of peptide combinatorial library

A peptide combinatorial library was constructed according to a known method (Pinilla, C. et al., Bio techniques, 13, 901 (1992)).

When a library consisting of hexa-peptides were synthesized, a predetermined amino acid residue was assigned to a specific one of the six positions while the other five positions were occupied by any of 19 kinds of amino acids (exclusive of cystein). Supposing that a specific amino acid residue at a determined position and other amino acid residues at the other positions in the peptides of six amino acid residues were represented by O and Xs, respectively, the peptides were expressed as $OX_2X_3X_4X_5X_6$, $X_1OX_3X_4X_5X_6$, ... $X_1X_2X_3X_4X_5O$. That is to say, in peptides of six amino acid residues, 19 kinds of amino acids are specified at each amino acid position while non-specified positions were occupied by any of amino acids exclusive of cystein to construct libraries of peptides, which amount to 114 (6×19) combinations.

Step 2: Primary search for peptide sequences

To select the peptides which can combine with VEGF from the combinatorial peptide libraries, the following experiment was conducted.

On a 24-well plate (Costar Co), HUVEC (human umbilical

vein endothelial cells) (Clonetics) were cultured in a medium at a density of 5×10^4 cells/well for one day. Then, the cells were transferred to a medium (Medium 199/25 mM HEPES, pH 7.4/0.1% bovine serum albumin) containing 0.2 ng of ^{125}I -labeled VEGF and various concentrations of peptides or combinatorial peptide libraries and reacted at 4°C for 3 hours. The cells were washed twice with the same medium and once with PBS containing 0.1% albumin to remove the labeled VEGF which still remained uncombined. Thereafter, to quantify the labeled VEGF which bound to receptors present on the surface of the vascular endothelial cells, the cells were treated with 0.5 ml of a lysis solution (20 mM Tris-HCl, pH 7.4/1% TritonX-100) for 20 min, followed by measuring the resulting lysate for radioactivity with the aid of a γ -counter.

With reference to Fig. 2, percent inhibition of combinatorial peptide libraries against the binding of VEGF to its receptors is represented according to amino acid residues on each position of the hexa-peptide when combinatorial peptide libraries are used at a concentration of 0.33 nM/peptide. Based on these results, 3-5 amino acids were selected for each position. As seen, the most potent activity antagonistic to VEGF was observed when lysine or arginine occupied all positions of the peptide.

Step 3: Secondary search for peptide sequences

Based on the primary search results, two pools of

peptides were synthesized as seen in Tables 2 and 3. Again, these two combinatorial peptide libraries were examined for the antagonistic activity against the binding of VEGF to its receptors and the results are given in Figs. 3a and 3b.

5 First, in order to make the first secondary pools of peptides, the two carboxyl terminal positions were specified by selected amino acids while the other positions were allowed to be occupied by the amino acid residues selected through the secondary selection in equal ratios. Amounting
10 to 375 sequences in total, this first secondary pool of peptides was composed of 15 combinatorial peptide libraries according to the combination of the two carboxyl terminal amino acids.

As in the primary search, this first secondary pool of
15 peptides was examined at various concentrations (0.01, 0.05, 0.1, and 1 μ M/sequence) for the antagonistic activity against VEGF binding. With reference to Fig. 3a, percent inhibition of peptide combinatorial libraries against the binding of VEGF to its receptors is represented according to
20 amino acid residues on the fifth and the sixth positions of the hexa-peptide when the first secondary pool of peptides were used at a concentration of 0.1 μ M/peptide. As seen in Fig. 3a, the peptide sequences which inhibited the binding with the highest efficiency were those with arginine or
25 histidine at their sixth position and arginine or lysine at their fifth position.

Next, the second secondary pools of peptides was

constructed by taking advantage of the results obtained through the two selection procedures. The first, the fourth, and the sixth positions from the amino end were specified by predetermined amino acids while the amino acid residue data was utilized for the other three positions, as shown in Table 3.

Combination of the specified amino acid residues resulted in 15 combinatorial libraries, each consisting of 50 sequences. The antagonistic activity of the peptides was examined at various concentrations (0.1, 0.25, and 1 μM /sequence). With reference to Fig. 3b, percent inhibition of combinatorial peptide libraries against the binding of VEGF to its receptors is represented according to amino acid residues on the first, the fourth, and the sixth positions of the hexa-peptide when the second secondary pool of peptides were used at a concentration of 0.25 μM /peptide. As seen in Fig. 3b, the most potent antagonistic activity to the binding of VEGF to its receptors was found in the sequences in which arginine occupied the first, the fourth, and the sixth positions all.

Step 4: Separation of peptide sequences

A mixture of the most effective peptides selected from the second secondary pool was separated through a C_{18} reverse-phase column into six fractions by the retention time in the column. Again, each of the six fractions was examined for the influence on the binding of VEGF to its

receptors at various concentrations (0.5, 1, 2, 5, 10, and 25 μ M/peptide). The results are given in Fig. 4, which shows curves plotted by the percent inhibition versus concentration of the peptide fractions.

5 In the curves, the fraction 1 has the highest antagonistic activity. Also, the fraction 1 was identified to contain no tryptophan as no absorbance at UV₂₈₀ was detected. Qualitative analysis of its amino acid sequences proved the presence of arginine, lysine, and histidine.

10 Therefore, the data obtained thus far demonstrated that the six-amino acid sequences with arginine at all of the first, the fourth and the sixth positions are the most antagonistic to the binding of VEGF to its receptors while the most effective candidates for the other positions can be
15 narrowed to two or three amino acid residues, e.g., arginine, lysine, and histidine for the second position, lysine and arginine for the third position, and arginine and lysine for the fifth position.

Resulting from the combination of the candidates, 12
20 peptides which were specified at the three positions by arginine were synthesized as shown in Table 4, followed by separating each peptide through a C₁₈ reverse-phase column.

Step 5: Tertiary search for peptide sequences

25 The sequence of each peptide was indirectly decided through the analysis of amino acid compositions and its concentration was determined, after which 12 peptides were

assayed for antagonism toward VEGF binding at various concentrations (1, 4, 10, and 20 μM /sequence). The results are given in Fig. 5. As seen in Fig. 5, three peptides, Sequences 1, 2, and 3, were found to inhibit the binding of VEGF to its receptors at highest efficiency with IC_{50} values of 2, 3.4, and 3.8 μM /sequence, respectively.

With reference to Fig. 6, there are shown quantitative results for the binding of I^{125} -labeled VEGF and I^{125} -labeled bFGF (basic fibroblast growth factor) to their receptors in the presence of various peptides. Despite a high concentration (10 μM) of a control peptide consisting of only lysine, no inhibition was observed between the labeled VEGF and the its receptors, showing that the inhibitory activity of the three peptides determined above does not result from positively charged amino acids, but is attributed to specific amino acid sequences. When it is taken into account that none of the three peptides inhibit the binding of bFGF, an angiogenic factor similar to VEGF, to its receptors, it is proven that they are specific for VEGF only.

EXAMPLE 3: Characterization of Peptides Screened from Combinatorial Peptide Libraries

There are various possible mechanisms to inhibit the binding of VEGF to its receptors. For instance, an inhibitor may be combined with either VEGF or one or more of its receptors, thereby inhibiting the interaction between

VEGF and the receptor.

To verify the postulated mechanism in which the screened peptides might be associated directly with VEGF so as to inhibit VEGF from binding to its receptors present on the surface of vascular endothelial cells, the following experiments were conducted. Each of the hexa-peptides was fixed on a 96-well ELISA plate at a concentration of 100 ng/well in such a manner that a 20% acetic acid solution containing each peptide was dried in air. The plate was treated three times with a phosphate buffered saline containing 0.1% albumin for 3 min each time. A solution of ^{125}I -labeled VEGF in the same phosphate buffered saline was added to the plate at a concentration of 0.2 ng/well and incubated at 37°C for 1 hour to associate the labeled VEGF with the peptides. To remove the peptides remaining unassociated, the plate was washed four times with the same phosphate buffered saline for 3 min per each time, followed by measuring radioactivity with the aid of a γ -counter. As for the non-specific binding, it was determined by incubation in the presence of either a mixture of the labeled VEGF and non-labeled VEGF or a mixture of the fixed peptide and unfixed peptide in a molar ratio of 1:100. This was set as a negative control.

With reference to Fig. 7, radioactivity measured from ^{125}I -labeled VEGF associated with fixed peptides is plotted versus molar ratios of a competitor, such as non-labeled VEGF or free peptides, to its counterpart. As seen in the

curves of Fig. 7, the radioactivity is in inverse proportion to the molar ratio, which demonstrates that the peptides directly associate with VEGF. The dissociation constant (KD) between VEGF and each peptide was determined using the
5 IC₅₀ value according to the following formula (De Blasi, A. et al., Trends Pharmacol. Sci., 10, 227 (1989)).

$$KD = IC_{50} - [\text{non-labeled Competitor}]$$

10 The dissociation constants determined using the formula were 5 μ M for Sequence 1, 2 μ M for Sequence 2, and 22 μ M for Sequence 3. From these results, it is apparent that all of the three peptides screened from the peptide combinatorial libraries associate with VEGF directly and
15 specifically.

Turning to Fig. 8, there are shown radiation quantities measured from ¹²⁵I-labeled VEGF associated with a fixed sequence in the absence of and in the presence of competitors. As seen in the histograms of Fig. 8, the
20 binding of VEGF to a sequence is almost completely prevented in the presence of excess amounts of the free three competitors. These results make it possible to postulate that Sequences 1, 2, and 3 have identical or overlapped binding domains on VEGF.

25 In order to verify this postulation, Sequence 1 (RRKRRR) was examined for the common binding domain on VEGF because it inhibited the binding of VEGF to its receptors at

the highest efficiency. First, appropriate primers were synthesized and used to amplify various cDNAs coding for VEGF₁₆₅, VEGF₁₂₁, VEGF₈₋₁₂₁, VEGF₁₀₉, and VEGF₈₋₁₀₉ while a human liver cDNA library (Clontech) served as a template. After
5 being inserted to plasmid pRSET A (Invitrogen), the cDNAs were sequenced. The resulting five pRSET A vectors, each having one of the five cDNAs, were introduced into an expression strain (BL21(DE3)pLysS) containing T7 RNA polymerase. Culturing these transformed cells produced
10 various VEGFs as inclusion bodies which were then separated at a purity of 90% or higher using a method reported previously (Siemeister, G. et al., Biochem. Biophysics. Res. Commun., 222, 249 (1996)). The purified VEGFs were quantified by use of a protein assay reagent (Bio-Rad) and
15 all found to bind to VEGF receptors and exert growth hormone activity on HUVEC. Each of the purified VEGFs was labeled with ¹²⁵I (0.5 mCi/5 µg of protein) with the aid of IODO-Bead (Pierce). For use, each labeled protein was quantified by ELISA (enzyme-linked immunosorbent assay) using a mouse
20 monoclonal anti-VEGF antibody (R&D systems).

Each peptide was examined for antagonistic activity against VEGF in the same manner as in Example 2. Referring to Fig. 9, the radioactivity measured from ¹²⁵I-labeled VEGF associated with fixed Sequence 1 (RRKRRR) is plotted in the
25 absence of competitors and in the presence of competitors for various VEGF isomers. From the fact that Sequence 1 (RRKRRR) inhibited not only the binding of labeled VEGF₁₆₅ to

its receptors, but the binding of the heparin-binding domain-deficient, labeled VEGF₁₂₁ to its receptors, it can be concluded that the heparin-binding domain of VEGF is independent of the association between the peptides and VEGF.

5 When it was taken into account that the other VEGF isoforms, VEGF₈₋₁₂₁, VEGF₁₀₉, and VEGF₈₋₁₀₉, were not inhibited from binding to their receptors by Sequence 1, the amino and the carboxyl ends of the VEGF₁₂₁ were believed to play a key role in binding the peptide to VEGF.

10

EXAMPLE 4: Assay for Inhibitory Activity of Peptides
Selected through the Tertiary Search Against VEGF-Stimulated
Vascular Endothelial Cell Growth

15 An examination was made in order to determine whether the peptides screened from the peptide combinatorial libraries, Sequence 1, Sequence 2, and Sequence 3, inhibit the VEGF-induced DNA synthesis of HUVEC.

20 In a gelatin-coated 48-well plate (Nunc) HUVEC were cultured at a density of 10^4 cells/well at 37°C for one day, followed by washing the cells three times with serum-free medium 199. After being added with culture media containing VEGF (10 ng/ml, R&D systems) and various concentrations of the screened peptides, the cells were cultured at 37°C. After 24 hours of culturing, the cells were added with
25 [methyl-³H] thymidine (0.5 µCi/well) and cultured for an additional one day. Before quantification of the radioactivity used for the DNA synthesis with the aid of a

liquid scintillation counter, the cells were washed with PBS containing 0.1% albumin, treated with 0.4 N NaOH at room temperature for 20 min for cell lysis, and neutralized with 2 N HCl. In order to determine the toxicity of the peptides, HUVEC were examined as in above using excess peptide (100 μ M) in the absence of VEGF.

With reference to Fig. 10a, percent inhibition against DNA synthesis is plotted versus peptide concentration. As seen in Fig. 10a, all of the screened peptides inhibit VEGF-induced DNA synthesis of HUVEC in dose-dependent patterns with IC₅₀ values ranging from 10 to 20 μ M.

With reference to Fig. 10b, the radioactivity of the thymidine inserted into the genome of HUVEC is measured in the absence of no peptides and in the presence of 100 μ M of each of the three peptides. The histograms of Fig. 10b show that the peptides have no direct influence on the growth of HUVEC and therefore lead to the conclusion that the peptides do not exert their inhibitory activity directly to cells.

Based on the results of the above experiments, it was concluded that the three peptides block the binding of VEGF to its receptors to specifically inhibit the growth of endothelial cells induced by VEGF.

EXAMPLE 5: Assay for Inhibitory Activity of Selected Peptides Against Angiogenesis Induced by VEGF or Human Cancer Cells Secreting VEGF.

To assay the peptides for anti-angiogenic activity,

egg CAM (chorioallantoic membranes) were examined as to whether the peptides inhibit the angiogenesis induced by VEGF.

Fertilized eggs (Pulmuwon, Korea) were incubated at 37°C under a humidity of 90%. After three days of culturing, the eggs were deprived of about 2 ml of albumin. After four days, eggs were partially deprived of the sheath to make a window with a size of 2 x 2 cm.

After VEGF (10 ng/egg) was mixed with various amounts of peptides or other samples, 3 µl of each mixture was dropped onto ¼ fraction pieces of thermanox coverslips (Nunc) and dried. The pieces were placed on CAM of 9-day embryonic eggs.

Two days later, the samples were independently observed under anatomical microscopes by two different persons to determine whether new blood vessels are induced by the dropped samples or not. In this regard, the experiment was repeated at least three times using 10 or more eggs per sample and the results are given in Table 5, below.

<TABLE 5>

SAMPLE	Angiogenic activity eggs/total eggs	Angiogenic activity(%)	P ^a
WATER	3/28	10.8(1.4) ^b	
VEGF(10ng)	9/27	33.6(3.8)	0.004
VEGF+RRKRRR(1µg)	4/26	15.6(5.1)	0.245
VEGF+RKKRKR(1µg)	4/26	15.6(4.5)	0.271

VEGF+RRRRRR (1µg)	4/26	15.6 (5.1)	0.245
VEGF+KKKKKK (1µg)	8/25	32.6 (12.2)	0.038
VEGF+protamine (1µg)	5/26	18.8 (4.1)	0.128

^a determined by comparing the values between water sample and other samples by use of student's t-test, statistically significant in the case of $p < 0.05$.

^b standard deviation

5

As seen in Table 5, VEGF was found to induce angiogenesis at a proportion of 33.6% in the italic model test. This angiogenic activity was effectively reduced to about 15.6% when treating egg samples with the peptides (1 µg/egg), along with VEGF and to about 18.8% when treating egg samples with protamine (50 µg/egg), known as an anti-angiogenic factor, along with VEGF. However, a control peptide (KKKKKK), which was not selected in spite of its similar properties to those of the screened peptides, did not show anti-angiogenic activity as it induced angiogenesis at a proportion of about 32.6%.

To confirm the test results obtained from egg CAM, an experiment was performed using rabbit corneal domes for *in vivo* angiogenesis testing. New Zealand male rabbits weighing 3 kg (SLC, Japan) were subjected to intramuscular ketamine anesthesia (44 mg/kg), followed by dissecting the corneal domes to a length of 3 mm by use of an operating knife (Bard-Parker #11). VEGF (10 ng, R&D systems) was dropped, alone or in combination with 1 µg of a peptide of an amino acid sequence EEFDDA or Sequence 1 (RRKRRR), onto a

Thermanox coverslip (Nunc) and dried under germ-free conditions, after which the coverslips were placed on the dissected areas which were then observed for natural healing. 6 rabbits were used per test group within which all animals were observed to show similar results. 16 days after the operation, angiogenesis was obviously observed and photographs were taken of blood vessels newly formed in the corneal dome (Nikon, FS-2, Japan). As seen in photographs of Fig. 11, the control peptide EEFDGA had no influence on the VEGF-induced angiogenesis in the rabbit corneal dome (Fig. 11C) while the angiogenesis which was obviously observed in the test group treated with VEGF only (Fig. 11A) was completely inhibited in the test group treated simultaneously with both Sequence 1 RRKRRR and VEGF (Fig. 11B).

The results from the above two experiments and Fig. 10b, taken together, demonstrate that the screened peptides have no direct influence on the growth of vascular endothelial cells, but block the binding of VEGF to its receptors present on the surface of vascular endothelial cells, thereby inhibiting the VEGF-induced angiogenesis *in vivo*.

An experiment using egg CAM was carried out to confirm the inhibitory activity of the screened peptides against the angiogenesis induced by cancer cells, which secrete VEGF. Fertilized eggs were deprived of albumin and windows were formed on the eggs as in above. 10^5 cells of HT1080 (human

fibrosarcoma) were mixed with 7.5 μ g of Type I collagen (rat tail, Beckton Dickinson, USA) within a volume of 5 μ l and dropped onto $\frac{1}{4}$ fraction pieces of Thermanox coverslips to give collagen sponges. After being covered with the collagen sponges, 10-day embryonic egg CAM was incubated at 37°C for 3 days. Blood vessels induced by the cancer cells were observed and photographed as in above (see Table 6 and Fig. 12).

10 <TABLE 6>

SAMPLE	Angiogenic activity eggs/total eggs	Angiogenic activity(%)	P ^a
No treatment	5/27	18.5(2.1)	
Cancer cell	18/24	76.0(8.5)	0.011
Cancer cell+RRKRRR(0.1 μ g)	8/22	36.0(8.5)	0.160
Cancer cell+RKKRKR(0.1 μ g)	10/23	43.0(9.9)	0.141
Cancer cell+RRRRRR(0.1 μ g)	10/24	41.0(7.1)	0.098
Cancer cell+KKKKKK(0.1 μ g)	14/22	59.0(1.4)	0.002

When having been cultured in egg CAM, human fibrosarcoma cells, which secrete VEGF, showed a typical "spokewheel" structure (10^5 cells/egg, 76%). It was observed that when being treated with the screened peptides (100 ng/egg, 36-43%) and the cancer cell, simultaneously, the egg CAM did not undergo angiogenesis. However, the control peptide did not exhibit inhibitory activity with a

statistical significance against the cancer cells (100 ng/well, 59%).

EXAMPLE 6: Assay for Direct influence of Screened Peptides on Human Fibro-Sarcoma Cell Line.

The following experiment was carried out to determine whether peptides antagonistic to VEGF have direct influence on the growth of human fibro-sarcoma cells. After being cultured for one day in a 96-well plate (Nunc), the cancer cells were added with DMEM containing various concentrations of the screened peptides and incubated. To the cells remaining alive after 3 days of incubation, 20 μ l of tetrazolium dye (Cell Titer 96 Non-Radioactive Proliferation assay kit, Promega) was added, followed by incubation at 37°C for 4 hours. The formazan produced by the viable cells was dissolved in 0.2 ml of dimethyl sulfoxide (DMSO) quantified by the absorbance at 570 nm. The absorbance attributed to formazan is proportional to living cells.

With reference to Fig. 13, cell viability of human fibroblast sarcoma cells is plotted versus concentrations of peptides. As seen in Fig. 13, all the screened peptides had no direct influence on the growth of human fibro-sarcoma cells.

According to the results obtained from this experiment and Example 5, it is apparent that, without direct influence on the growth of human fibro-sarcoma cells, the screened peptides inhibit the angiogenesis induced by the cancer

cells by specifically blocking the binding of VEGF secreted from the cancer cells to its receptors present on the surface of vascular endothelial cells.

5 EXAMPLE 7: Assay for Effect of Screened Peptide on Human Colon Carcinoma Cells (HM7).

It was reported that the acquirement of angiogenic ability is crucial to the progression of cancer and indispensable for the continuous growth of cancerous tissues (Hanahan, D. et al., Cell, 86, 353 (1996); Skobe, M., et al., Nature Med., 3, 1222 (1997). Also, the screened peptides were found to effectively inhibit angiogenesis in vivo. With this information, the following experiment was made to determine whether the screened peptides effectively inhibit the growth and metastasis of cancer cells. 5x10⁶ cells of HM7 were added, together with 0.5 µg/µl of an amino acid sequence EEFDDA or Sequence 1 (RRKR^{RR}), to a serum-free DMEM and then introduced into male mice which were 4 weeks old (athymic nude mice, BALB/c/nu/nu, Charles River, Japan) by subcutaneous injection. From the next day, a solution of each peptide in PBS (0.5 µg/100 µl/day) was subcutaneously injected to the mice for 15 days. Sizes of the tumors thus formed were periodically measured while tumor volumes were calculated according to the following formula:

$$\text{Tumor Size} = 0.5 \times (\text{Diameter})^2 \times \text{length}$$

In order to conduct an experiment concerning the metastasis of cancer cells to the liver, cancerous cells were transplanted into the spleen. In this regard, after being anesthetized with diethyl ether, 4-week-old male mice (athymic nude mice, BALB/c/nu/nu, Charles River, Japan) underwent flank incision. To the spleen, 100 μ l of a mixture containing 10^6 cells of HM7 (human colon carcinoma cell line) and 0.5 μ g/ μ l of amino acid sequence EEFD~~DA~~, KKKKKK, or Sequence 1 (RRKR~~RR~~) was slowly injected, followed by the subcutaneous injection of each peptide for three weeks as in above. Four weeks after the injection, the liver was excised from each mouse and measured for weight and the size and number of metastatic nodules formed. Each test group was composed of 6-7 mice. In a student's t-test, a p value less than 0.05 was regarded as being statistically significant. In the test using tetrazolium dye (cell titer 96 Non-radioactive Proliferation Assay Kit, Promega), each peptide was evaluated to have no influence on the growth of the cancer cells (5×10^3 cells/well), so that the possibility that the toxicity of the peptide themselves might inhibit the growth and metastasis of cancer cells could be excluded.

With reference to Fig. 14, changes in tumor size are recorded over the time period of injection. After 15 days of subcutaneous injection, the sequence EEFD~~DA~~ exhibited no effects whereas the peptide RKR~~RR~~ of Sequence 1 decreased the tumor size by about 28% compared to the control (PBS). Turning to Fig. 15, the numbers of metastatic nodules and

liver weights after 14 days of injection are shown according to injected materials. No difference in metastasis of cancer cells could be found between the groups to which tumor was injected alone and together with the sequence
5 EEFDDA. A weak inhibitory activity was observed in the group to which the sequence KKKKKK was injected (about 80% of the control group to which only PBS was injected). In contrast, high inhibitory effects were found from the test group to which the peptide RRKRRR of Sequence 1 was injected
10 as the test group was only 16% and 33% of the control group in the number of metastatic nodules and the weight of the liver, respectively. Therefore, it is apparent that the screened peptides shield the signal transduction of VEGF to inhibit the growth and metastasis of malignant tumors.

15
When the peptides of Sequences 1, 2, and 3 are to be clinically used, parental routes are preferred. They are injected at an effective dose of 0.1-100 µg/kg and preferably at a dose of 0.5-10 µg/kg once a day for 2-3
20 weeks.

The peptides of Sequences 1, 2, and 3 were tested for acute toxicity through the following experiment.

25 EXAMPLE 8: Acute Toxicity Test On Rat Upon Non-Oral Administration

Using specific pathogen-free (SPF) SD rats which were

six weeks old, the peptides of Sequences 1, 2, and 3 were tested for acute toxicity. Suspensions of the peptides in 1 ml of PBS were administered at a dose of 1 mg/kg to the rats, which were grouped in twos, by intramuscular injection.

5 After the administration, the animals were observed as to whether they died, which clinical symptoms they showed and how their weights were changed; and serologically and serobiochemically tested. An autopsy was made of the rats with the naked eye to observe whether their abdominal and
10 thoracic organs were damaged. Neither sudden death nor noticeable clinical symptoms were detected in any of the animals administered with the peptides of interest. In addition, no toxicity signs were observed in terms of weight change, serological tests, serobiochemical tests, and corpse
15 examination. Further, *italic* cytotoxicity tests revealed that the peptides of Sequences 1, 2, and 3 damage neither of endothelial cells, human fibro-sarcoma cells nor human colon carcinoma cells. In consequence, the peptides of Sequences 1, 2, and 3 caused no toxic changes to the rats at a dosage of
20 1 mg/kg and thus, were found to be safe compounds with a lethal dose (LD50) of at least 1 mg/kg when being administered via a non-oral route.

As described hereinafter, the peptides of the present invention are associated with VEGF to block its binding to
25 receptors present on the surface of vascular endothelial cells, thereby inhibiting the hormonal activity of VEGF, which is related to angiogenesis. Because cancer cells

secrete VEGF to generate new blood vessels for their growth and metastasis, the peptides of the present invention are also useful to inhibit the growth and metastasis of cancer cells. Therefore, the peptides of the present invention can
5 be used as therapeutics for angiogenesis-related diseases, including cancer, diabetic retinopathy, rheumatoid arthritis, etc, thanks to superior ability to inhibit the binding of VEGF to its receptors. Additionally, anti-angiogenic therapies for cancer using the peptides of the present
10 invention inhibit the growth of host normal cells (vascular endothelial cells), but not cancer cells themselves, so that they are expected to overcome the problems of conventional therapies for cancer, which are due to the versatility and resistance of cancer cells

15 The present invention has been described in an illustrative manner, and it is to be understood that the terminology used is intended to be in the nature of description rather than of limitation. Many modifications and variations of the present invention are possible in
20 light of the above teachings. Therefore, it is to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.